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Environmental Nitroarenes: An Attempt to Understand Their Mutagenic and Carcinogenic Properties

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ENVIRONMENTAL NITROARENES: AN ATTEMPT TO UNDERSTAND THEIR MUTAGENIC AND CARCINOGENIC PROPERTIES

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16. ABSTRACT

This report will deal primarily with subsequent advances and an attempt to place the emerging knowledge into perspective. Nitroarenes have been found in xerographic toners, typewritter ribbons, and cigarette smoke condensate (e.g., mainstream). The reason for the present concern about the possible health hazards of these chemicals is derived from the realization that they are present in diesel emissions, in fly ash derived from coal conversion processes and in the sidestreams of cigarette smoke. Thus, they are not only ubiquitous in the atmosphere but may also be present in high concentrations in confined spaces, such as the office and home environment, primarily as the result of cigarette smoking. Literally hundreds of nitroarenes have been detected in diesel emissions and in the ambient atmosphere. It is now realized that nitroarenes are perhaps ubiquitious as a result of the facile nitration by oxides of nitrogen (in the presence of traces of acids) of polycyclic aromatic hydrocarbons (PAHs) emitted during the course of incomplete combustion processes. Obviously, the recognition of hundreds of environmental nitroarenes means that it will not be practical to prepare sufficient quantities of each of these and to determine their mutagenic and carcinogenic potentials. Accordingly, our approach has been to study structural commonalities which might be useful in predicting biological properties. Some of our preliminary findings are reported herein.

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Chapter 3

ENVIRONMENTAL NITROARENES: AN ATTEMPT TO UNDERSTAND THEIR MUTAGENIC AND CARCINOGENIC PROPERTIES

H. S. Rosenkranz, E. C. McCoy, R. Mermelstein, and G. Klopman

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I. INTRODUCTION

There has been an explosion of information related to the environmental distribution and the biological properties (primarily genotoxicity, mutagenicity, and carcinogenicity) of the nitroarenes. Recently, we have reviewed[1] the mutagenic and carcinogenic properties of these chemicals and hence, the present report will deal primarily with subsequent advances and an attempt to place the emerging knowledge into perspective.

Even though nitroarenes have been found in esoteric places (e.g., xerographic toners from which they have now been eliminated),[2,3] typewriter ribbons,[4] and cigarette smoke condensate (e.g., mainstream),[5] the reason for the present concern about the possible health hazards of these chemicals is derived from the realization that they are present in diesel emissions, in fly ash derived from coal conversion processes and in the sidestreams of cigarette smoke.[1,6,7] Thus, they are not only ubiquitous in the atmosphere but may also be present in high concentrations in confined spaces, such as the office and home environment, primarily as the result of cigarette smoking.[8] Literally hundreds of nitroarenes have been detected in diesel emissions and in the ambient atmosphere.[9-12] It is now realized that nitroarenes are perhaps ubiquitous as a result of the facile nitration by oxides of nitrogen (in the presence of traces of acids) of polycyclic aromatic hydrocarbons (PAHs) emitted during the course of incomplete combustion processes.[6,7,13-17] Obviously, the recognition of hundreds of environmental nitroarenes means that it will not be practical to prepare sufficient quantities of each of these and to determine their mutagenic and carcinogenic potentials. Accordingly, our approach has been to study structural commonalities which might be useful in predicting biological properties. Some of our preliminary findings are reported herein.

II. MUTAGENICITY FOR *SALMONELLA TYPHIMURIUM*

Most nitroarenes exhibit direct-acting mutagenicity for *Salmonella typhimurium* (Table 1). The activity is of the frameshift variety as evidenced by the selectivity for strains TA1538 and TA98.[1] For most nitroarenes, no exogenous metabolic activation is required for expression of the mutagenicity (Table 1). Several features are noteworthy: (1) some members of the group, such as the dinitropyrenes, are among the most mutagenic species reported in the literature; (2) the mutagenicity appears to increase with the extent of nitration; and (3) extensive differences in mutagenic potencies exist among the various isomers (Table 1).

Even though nitroarenes appear to be direct-acting mutagens for *Salmonella typhimurium*, their mutagenicity is dependent upon reduction to the corresponding arylhydroxylamines[1,18-26] and in some instances their subsequent esterification[27] to arylhydroxamic acid esters (Figure 1). This was demonstrated by the characterization of derivatives of *Salmonella typhimurium* tester strains deficient in some of the required enzymes.

Thus, *Salmonella* strains blocked in the classical nitroreductase, the enzyme which is required for the demonstration of the expression of the mutagenicity of nitrofurans and nitroimidazoles, are also not responsive to the mutagenicity of some nitroarenes (Table 2).[1] The fact, however, that some nitroarenes (such as 1,8-dinitropyrene) are fully mutagenic in this strain indicates the existence of additional nitroreductases. The specificity of the enzymic block in TA100 as well as TA98 (i.e., strains TA100NR and TA98NR, respectively) was ascertained by demonstrating that nitrosoarenes and arylhydroxylamines are fully active in these strains (Table 3). This was verified further by demonstrating, using HPLC techniques, that such strains did not in fact reduce nitroarenes.

Additional strains insensitive to the mutagenicity of other mononitroarenes and dinitroarenes were also isolated (e.g., TA98/1,8-DNP_6, Table 2). Initially it was thought that all of these strains were also devoid of nitroreductases specific for these nitroarenes.[19] However, further studies revealed that some of these strains even though not mutagenized were still

Table 1
MUTAGENICITY OF NITROARENES FOR *SALMONELLA TYPHIMURIUM* TA98

Chemical	Mutant/nmole −S9	Mutant/nmole +S9	Ref.
1-Nitronaphthalene	0.05		79
2-Nitronaphthalene	0.2		79
2-Methyl-1-nitronaphthalene	0		80
1-Methyl-2-nitronaphthalene	0.2		80
3-Methyl-2-nitronaphthalene	1.0		80
1,3-Dinitronaphthalene	0.9		79
1,5-Dinitronaphthalene	3.3		79
1,8-Dinitronaphthalene	0		79
2,4-Dinitro-1-naphthol	0.2		79
1,3,6,8-Tetranitronaphthalene	0.2		79
3-Nitro-1,8-naphthalic anhydride	2.6		79
5-Nitroacenaphthene	2.5		59
2-Nitrofluorene	14		81
3-Nitro-9-fluorenone	383		82
2,7-Dinitrofluorene	471		81
2,7-Dinitro-9-fluorenone	1,459		81
2,4,7-Trinitro-9-fluorenone	2,125		81
2,4,5,7-Tetranitro-9-fluorenone	860		81
2-Nitroanthracene	892		83
9-Nitroanthracene	0.5		84
2-Nitrophenanthrene	0.5		84
1-Nitrofluoranthene	74		84
3-Nitrofluoranthene	5,439		84
7-Nitrofluoranthene	544		84
8-Nitrofluoranthene	11,125		84
1-Nitropyrene	453		18
2-Nitropyrene	2,225		84
1,3-Hydroxynitropyrene	7,170		9
1,3-Dinitropyrene	144,760		18
1,6-Dinitropyrene	183,570		18
1,8-Dinitropyrene	254,080		18
1,3,6-Trinitropyrene	40,700		18
1,3,6,8-Tetranitropyrene	15,590		18
2-Nitrochrysene	0.6	27	84
5-Nitrochrysene	0.6	2.7	85
6-Nitrochrysene	269		84
7-Nitrobenz[*a*]anthracene	0.3	1.4	83
6-Nitrobenzo[*a*]pyrene	0	466	74
1-(or 3-)Nitrobenzo[*a*]pyrene	1,567		73
3-(or 1-)Nitrobenzo[*a*]pyrene	1,070		73
1-Nitrobenzo[*e*]pyrene	39		84
3-Nitrobenzo[*e*]pyrene	890		84
3-Nitroperylene	30	1,784	84
4-Nitrobenzo[*g,h,i*]perylene	0.6	1,925	84
7-Nitrobenzo[*g,h,i*]perylene	0.3	0.6	84
1-Nitrocoronene	2.8	7.0	84
1-Nitrocarbazole	0	0	86
2-Nitrocarbazole	10.3		86
3-Nitrocarbazole	0.2	0.8	86
4-Nitrocarbazole	0.01	0.06	86
5-Nitroquinoline	0.1		87
6-Nitroquinoline	0.05		87
8-Nitroquinoline	0		87

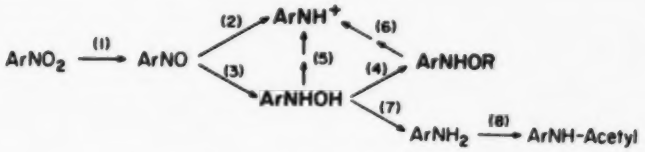

FIGURE 1 Biotransformation of nitroarenes in bacteria. (The bold-lettered intermediates are believed to be ultimate mutagens and/or DNA reactants.)

Table 2
THE MUTAGENIC RESPONSE OF *SALMONELLA* TA98 DERIVATIVES

Chemical	Revertants/nmol		
	TA98	TA98NR	TA98/1,8-DNP$_6$
1-Nitropyrene	453	35	199
1,3-Dinitropyrene	144,760	24,750	2,750
1,6-Dinitropyrene	183,570	190,900	45,890
1,8-Dinitropyrene	254,000	264,160	5,850
1,3,6-Trinitropyrene	40,700	36,300	25,640
1,3,6,8-Tetranitropyrene	15,600	10,400	14,000
Nitrazole	990	17	990
4-Nitroquinoline-1-oxide	126	124	106
2,7-Dinitro-9-fluorenone	1,459	184	137
2-Nitrofluorene	84	2	2
5-Nitroacenaphthene	5	1	0.5
4-Nitro-*o*-phenylenediamine	4	2	0.1

Table 3
MUTAGENICITY OF NITROARENES AND THEIR DERIVATIVES FOR NITROREDUCTASE-DEFICIENT *SALMONELLA TYPHIMURIUM*

Chemical	Revertants/nmol			
	TA100	TA100NR	TA98	TA98NR
1-Nitronaphthalene	1.0	0		
N-Hydroxy-1-aminonaphthalene	10.6	11.8		
2-Nitronaphthalene	1.3	0.1		
N-Hydroxy-2-aminonaphthalene	15.0	16.1		
2-Nitrofluorene			84	2
2-Nitrosofluorene			542	504
N-Hydroxy-2-aminofluorene			1175	1266

Note: NR denotes strains deficient in the "classical" nitroreductase.

capable of reducing the nitro group of the nitroarene to which they were insensitive. In addition, unlike TA98NR, the block in TA98/1,8-DNP$_6$ was not overcome by supplying the cells with preformed nitrosoarenes and arylhydroxylamines (Table 4). Only the model ultimate mutagen *N*-acetoxy-2-acetylaminofluorene was mutagenic in that strain (Table 4).

Table 4
MUTAGENICITY OF NITROARENES AND DERIVATIVES FOR *SALMONELLA TYPHIMURIUM* TA98/1,8-DNP$_6$

Chemical	Revertants/nmol	
	TA98	TA98/1,8-DNP$_6$
BMBA	14	17
Quercetin	0.9	1
1,8-Dinitropyrene	254,000	5,840
2-Nitrofluorene	84	2
2-Nitrosofluorene	542	79
N-Hydroxy-2-aminofluorene	1,175	31
N-Acetoxy-2-acetylaminofluorene	80	9

Note: BMBA, 7-Bromomethyl-12-methylbenz(a)anthracene.

Table 5
2-NITROFLUORENE METABOLITES IDENTIFIED IN EXTRACTS FROM *SALMONELLA* TESTER STRAINS

Strain	N-OH-2-AF	2-AF	2-AAF	N-Acetoxy-2-AAF
TA98	+	+ + +	+	+
TA98NR				
TA98/1,8-DNP$_6$	+	+	+	

Abbreviations: N-OH-2-AF, *N*-hydroxy-2-aminofluorene; 2AF, 2-aminofluorene; 2AAF, 2-acetylaminofluorene. Bacterial suspensions were incubated with 2-nitrofluorene (25 μg/ml) extracted and processed for chromatographic analysis as described in the legend of Figure 2.

This suggested that strain TA98/1,8-DNP lacks an esterification enzyme.[7] Subsequent studies indicated that the esterification enzyme was a transacetylase, the activity of which could be detected by HPLC analysis of cell extracts. Thus, in extracts of TA98 exposed to 2-nitrofluorene, the putative *N*-acetoxy-2-acetylaminofluorene could be detected while it was absent in extracts derived from TA98/1,8-DNP$_6$ (Table 5). The nature of this lesion was verified further by demonstrating that the mutagenicity of *N*-hydroxy-2-aminofluorene for TA98/1,8-DNP$_6$ was partially restored by the addition of PAPS, acetylCoA, or acetylphosphate; the latter two have been shown to form the *N*-acetoxy derivative nonenzymatically. In addition, incubation of *N*-hydroxy-2-acetylaminofluorene with these co-factors actually resulted in conversion to direct-acting mutagens (Table 6).

The activity of this transacetylase could be readily demonstrated using HPLC procedures with 2-aminofluorene serving as the acetyl acceptor. This activity was present in TA98 and TA98NR, but it was absent in TA98/1,8-DNP$_6$ (Figure 2) and similar strains.[7]

Based upon the results of the type of studies outlined above and the isolation and characterization of several hundred mutant strains not responsive to the mutagenicity of nitroarenes, a scheme unifying the experimental details was proposed (Figure 1).

Nitroreduction (Reaction 1) is catalyzed by several nitroreductases including (a) the classical nitroreductase, (b) the enzyme which catalyzes the nitroreduction of 4-nitroquinoline-1-oxide, and (c) the enzyme which acts on 1,8-dinitropyrene and reduces it such that it can be acted upon by the esterification enzyme. Reaction 3 is catalyzed by a *nitroso* reductase

Table 6
EFFECT OF ADDITION OF CO-FACTORS ON THE MUTAGENICITY OF *N*-HYDROXY-2-ACETYLAMINOFLUORENE

	Revertants/μg	
Additions	**TA98**	**TA98/1,8-DNP$_6$**
None	2.5	1.5
Acetylphosphate	9.6	11.1
AcetylCoA	25.7	14.9
PAPS	6.8	4.0

Note: It should be noted that assays were run in the absence of S9. Nonenzymic esterification of *N*-hydroxy-2-acetylaminofluorene was ascertained by HPLC. Mutagenicities were calculated from the linear portions of the dose response curves.

which is different from the classical nitroreductase as evidenced by the full activity of nitrosoarenes in strains TA100NR and TA98NR (Table 3). Depending upon the nature of the arene, nitrosoarenes and *N*-hydroxylaminoarenes can be converted nonenzymatically to the reactive nitrenium ion (Reactions 2 and 5). Some arylhydroxylamines, however, appear to require esterification to the arylhydroxamic acid esters (Reaction 4). Thus, nitroarenes such as 1,8-dinitropyrene and 2-nitrofluorene are almost entirely dependent upon this esterification, as evidenced by their lack of activity in strain TA98/1,8-DNP$_6$. Others such as 1,3,6,8-tetranitropyrene (which exerts activity in TA98/1,8-DNP$_6$, Table 2) may form arylhydroxylamines which are either potent electrophiles or become so following nonenzymic conversion to arylnitrenium ions (Reaction 5). It is believed that the arylhydroxamic acid esters derive their mutagenicity from a nonenzymic conversion to arylnitrenium ions (Reaction 6). Other nitroarenes (e.g., 1-nitropyrene) which exhibit 50% of their activity in strain TA98/1,8-DNP$_6$ (Table 2) probably form arylhydroxylamine intermediates (or their derived arylnitrenium ions) which are only half as effective as their corresponding hydroxamic acid esters in reacting with DNA. Finally, for some arylhydroxylamines, esterification appears to be a "detoxification" reaction as evidenced by an increased mutagenicity in strain TA98/1,8-DNP$_6$ (Table 7). In addition to these activating reactions, the arylhydroxylamines can be converted further to the nonmutagenic arylamines (Reaction 7) and these can be further acetylated to the *N*-acetylaminoarenes (Reaction 8). It is conceivable that this latter reaction is catalyzed by the same enzyme (a transacetylase) that is responsible for Reaction 4 (see above and Figure 2).

III. THE MOLECULAR BASIS OF THE MUTAGENICITY OF NITROARENES

As mentioned earlier,[1] nitroarenes are mutagenic for *Salmonella* tester strains TA1538, TA98, as well as TA100. The first two are indicator strains for frameshift mutations while the latter is a plasmid pKM101-containing strain derived from TA1535. Strain TA1535 is prototypic of strains used to detect base substitution mutations. Plasmid pKM101 codes for an error-prone DNA repair system which increases susceptibility to mutagens in strains

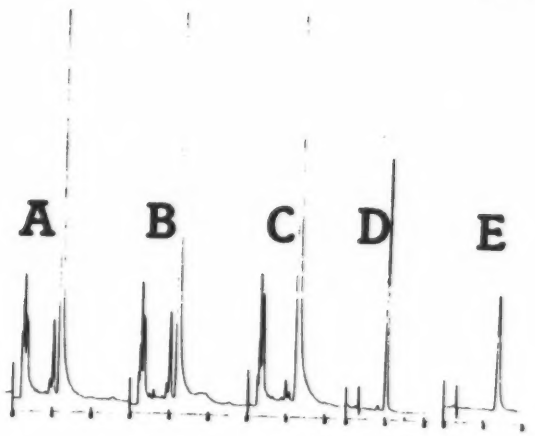

FIGURE 2. HPLC profiles of extracts from bacterial cells exposed to 2-aminofluorene (A) TA98, (B) TA98NR, and (C) TA98 1,8-DNP$_2$. Panels D and E represent the elution profile of 2-acetylaminofluorene and 2-aminofluorene, respectively. Note the great diminution in the peak representing 2-acetylaminofluorene (retention time: 4.0 min) in the extract derived from TA98 1,8-DNP$_2$ (C). Portions (2.5 ml) of bacterial suspensions (approximately 1.6×10^9 ml) of 0.1*M* 7 phosphate buffer, pH 7.4, containing 1.6% glucose) were incubated for 90 min at 37°C with 2-aminofluorene (final concentration: 25 μg/ml) whereupon the cells were harvested, washed, and resuspended in 1 ml of phosphate buffer. They were disrupted by sonication, an equal volume of cold acetone was added, and the mixture extracted twice with two volumes of methylene chloride. Dehydration was achieved by the addition of 1 g of sodium sulfate. The supernatant fluids were brought to dryness and resuspended in 100 μl of methanol. These preparations were analyzed with a Varian 5000 HPLC apparatus with a UV detector. The column used was a C_{18} μBondapak MCH$_{10}$ (0.4 × 30 cm) and isocratic elution was performed with 60% acetonitrile/water. The flow rate was 2 ml/min at 25°C.

Table 7
MUTAGENICITY OF 3,2′-DIMETHYL-4-NITROSOBIPHENYL

Strain	Revertants/μg
TA100	5.0
TA100.1n5.1,8-DNP$_2$ 1011	9.0
TA100.1n5.1,8-DNP$_2$ 1012	17.2
TA98	1.4
TA98.1,8-DNP$_2$	2.8
TA98NR.1,8-DNP$_2$	2.1

Note. All strains except for TA98 and TA100 are deficient in an esterification enzyme. Strain TA98NR.1,8-DNP$_2$ is also deficient in the classical nitroreductase.

Table 8
BASE SEQUENCE AT MUTATIONAL SITES OF *SALMONELLA* TESTER STRAINS

Tester strain	Mutational hot spot	Ref.
TA97	– C C C C C C – – G G G G G G –	57
TA98	– C G C G C G C G – – G C G C G C G C –	88
TA100	– C C* C – – G G* G –	89
TA96	– A A A A A – – T T T T T –	41
TA102	– G A* A A – – C T* T T –	41

harboring it.[29,41] The presence of pKM101 is, however, accompanied by a loss of mutagenic specificity.[90] Thus, while TA1535 is nonresponsive to the mutagenicity of most nitroarenes, TA100 is mutagenized by this group of chemicals. All of these strains share the fact that they contain guanine at their mutational targets (Table 8). This suggests that nitroarenes derive their mutagenicity from an ability to form adducts with the guanine moiety of DNA. Indeed biochemical studies have shown that such adducts are formed. Thus in *Salmonella* 1-nitropyrene forms almost exclusively *N*-(deoxyguanosin-8-yl)1-aminopyrene.[24] The mutagenic potency of arylhydroxylamine intermediates of 2-nitronaphthalene (or 2-aminonaphthalene), 2-nitrofluorene (or 2-aminofluorene), and 1-nitropyrene has been correlated with the extent of binding to the C-8 moiety of the DNA guanine.[23,34,35] A similar relationship has been established between some of these chemicals and their ability to induce bladder cancer.[35]

Although arylation of the C-8 of guanine appears to be important for biological activity, it must be mentioned that other targets exist as well. Thus the activated metabolite of 1-nitronaphthalene, *N*-hydroxy-1-aminonaphthalene, does not react at all at the C-8 position of the guanine, but rather it forms two other guanine adducts: 1-(deoxyguanosin-N^2-yl)-1-aminonaphthalene (60%) and 2-(deoxyguanosin-O^6-yl)-1-aminonaphthalene (30%).[35,36] In addition to forming the C-8 aryl derivative (50%), *N*-hydroxy-2-aminonaphthalene also forms 1-(deoxyguanosin-N^2-yl)-2-amino-naphthalene and 1-(deoxyadenosin-N^6-yl)-2-amino-naphthalene.[35,37] It must be pointed out, however, that this lack or decrease in the reactivity at the C-8 position of guanine is accompanied by greatly diminished mutagenicity of these naphthalene derivatives for both TA100 and TA98 (Tables 1 and 3).

IV. RELATIONSHIP BETWEEN THE ELECTROCHEMICAL REDUCTION OF NITROARENES AND THEIR MUTAGENICITY

Prior studies[1] (see above as well) have suggested that nitroreduction appears to be essential for the mutagenicity of most of the nitroarenes in *Salmonella*. Interest has focused on the properties of the nitropyrenes because (1) they are perhaps the most potent bacterial mutagens,[38] (2) they appear to be the most abundant nitroarenes in diesel emissions as well as in the ambient environment, (3) they were the first to be described in environmental specimens,[2,3,7] and (4) because they show interesting variations in the mutagenicity as results of position effects (Table 1).[38] Accordingly, when it became feasible to examine the polar-

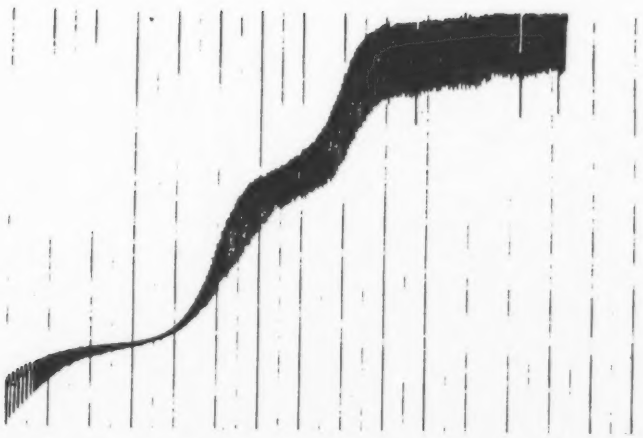

FIGURE 3 The polarographic reduction of 1,3-dinitropyrene. The specimen was dissolved in a 4:1 mixture of DMSO and 0.1 *M* sodium acetate, pH 4.5.

ographic reduction of nitroarenes, our interest was focused primarily on the properties of the nitropyrenes. We had two main purposes in mind when initiating the study:

1. To determine whether a relationship existed between the ease of electrochemical reduction and the mutagenic potencies, which in turn would indicate that nitroreduction was not only an essential step in the promutagenic event but also that the ease of nitroreduction might be a more important determinant of mutagenicity than the nature of the ultimate DNA adduct that was formed.
2. Hückel-type quantum mechanical calculations permit the determination of reduction potentials. It was, therefore, possible that if a correlation between reduction potential and mutagenic potency was found, this would enable the prediction of the mutagenic potencies of yet untested nitroarenes. This is an important objective in view of the presence in the environment of hundreds of nitroarenes of, as yet, undetermined mutagenicity.

The nitropyrenes studied could be reduced electrochemically using a dropping mercury electrode and a saturated calomel reference electrode. The number of reduction waves was equal to the number of nitro functions present in the molecule (Figure 3). A plot of the logarithm of the mutagenicity of the nitropyrene (log mutants per nanomole, see Table 9 for the data used) vs. the half wave reduction potential of the first reduction wave revealed a good correlation between mutagenic potencies and the ease of reduction (Figure 4, see also Figure 9). This indicates that the ease of reduction of the first nitro function is significantly correlated with the mutagenic potency. In turn, this suggests that with respect to the ultimate promutagenic event this appears to be a more important factor than the nature of the DNA adduct. As a matter of fact, the results show that the ease of nitroreduction is related directly to the extent of DNA adduct formation as evidenced by the linear relationship

Table 9
MUTAGENICITIES OF NITROPYRENES

Chemical	Revertants/nmol TA1538	TA98	TA100
1-Nitropyrene	85	453	84
1,3-Dinitropyrene	78,960	144,760	10,879
1,6-Dinitropyrene	61,100	183,570	5,426
1,8-Dinitropyrene	34,700	254,000	5,548
1,3,6-Trinitropyrene	20,300	40,700	1,256

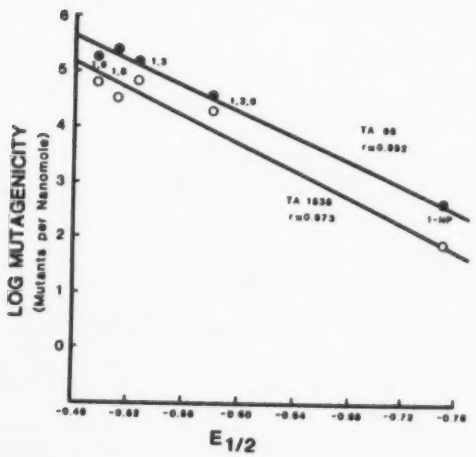

FIGURE 4 A plot of the logarithms of the mutagenicities of nitropyrenes in strains TA1538 and TA98 (data from Table 9) vs. the voltage of the half wave potential ($E^{1}/_{2}$) for the reduction of the first nitro group. The numbers near the experimental points refer to 1-nitropyrene (1-NP), 1,3-, 1,6-, and 1,8- to the corresponding dinitropyrenes and 1,3,6 to the trinitropyrene.

(r = 0.973) between the log of the mutagenicity in TA1538 and the voltage of the half wave potential (Figure 4). It has been noted that mutagenic potency of 1-nitropyrene and other nitroarenes in TA1538 has been related to the extent of adduct formation.[21,35] As a corollary, the results suggest that the nature of the adducts and the structural distortions they induced make only a minor contribution to the mutagenicity of nitroarenes. This can be deduced from the linearity of the plots of the logarithm of the mutagenicity in TA98 vs. the voltage of the half wave potential (Figure 4). Strain TA98 also contains plasmid pKM101 which codes for an error-prone DNA repair system. This repair enzyme magnifies mutagenic events when they are the result of major structural distortions. Indeed, the nitroarenes as a group appear to be more mutagenic in TA98 than in TA1538. However, the linearity (r =

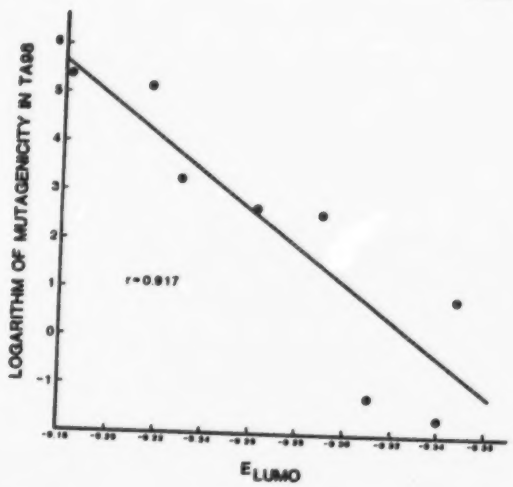

FIGURE 5 Plot of the logarithm of the mutagenicity of nitroarenes in TA98 (mutants per nanomole) against E_{LUMO}, the energy in the lowest unoccupied orbital. It should be noted that in addition to the data for the nitropyrenes the plot includes values for 1-nitronaphthalene, 2-nitronaphthalene, 2-nitrofluorene, 2,7-dinitrofluorene, and 2,7-dinitro-9-fluorenone, as well

0.992) of the relationship between TA98 and half wave potential (Figure 4) suggests either that the structural alterations induced by all of the nitroarenes tested are identical and therefore that they are cancelled out, or that the contribution of the structural distortions to the mutagenicity of even the plasmid-bearing strain is minimal.

In view of the linearity of the relationship between the voltage of the half wave potential and the logarithm of the mutagenicity, Hückel-type calculations evaluating electronic properties relevant to the ease of reduction based upon energies of the lowest unoccupied orbital (LUMO) were carried out. Indeed such calculations revealed a linear relationship between the value of the energies of LUMO and the half wave potentials, and therefore also between the logarithm of the mutagenicity and the LUMO energies (Figure 5). The findings suggest that theoretical calculations can be used to predict the mutagenic potencies of yet untested nitroarenes.

V. AN EMPIRICAL APPROACH TO DETERMINING THE STRUCTURAL DETERMINANTS OF THE MUTAGENICITY OF NITROARENES

As mentioned earlier, hundreds of nitroarenes have been recognized in the environment as well as in diesel emissions. Knowledge of the mutagenicity of the nitroarenes is restricted to only a fraction of these (Table 1). A visual inspection of the structural features of these molecules does not reveal a pattern which can explain the potent mutagenicity of some and the lack of activity of others. In order to determine features of the molecules which are determinants of the mutagenicity, we have studied the polarographic reduction of the ni-

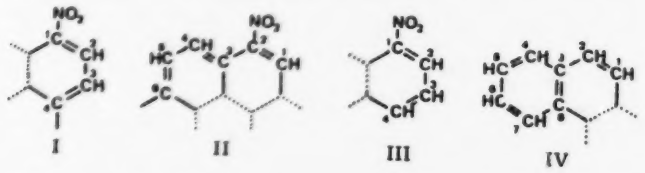

FIGURE 6. The two active (I and II) and the two deactivating fragments (III and IV) found by CASE to be relevant to the mutagenicity of nitroarenes.

troarenes (see above) since, should a relationship exist between ease of nitroreduction and mutagenicity, it would in turn justify an effort based upon quantum mechanical calculations that could lead to predictions of mutagenic potencies based upon theoretical considerations. Indeed such an approach seems feasible in view of the demonstration of a relationship between ease of nitroreduction and mutagenic potencies (Figure 4) and the corollary revelation of a relationship between mutagenicity and LUMO energies (Table 5). Another approach has recently become possible as a result of the development in this institution of CASE ("Computer Automated Structure Evaluation").[58] This innovative procedure consists of fragmenting the molecule under study into subunits composed of three or more heavy atoms (with their attached hydrogens); fragments derived from mutagenic nitroarenes are designated as positive while those derived from nonmutagenic molecules are designated as negative. The results are tabulated and analyzed statistically. Any significant deviation from a random distribution of subunits between the pools of fragments derived from active and inactive molecules is taken as an indication that the subunit is relevant to the property under study.[58] The analysis of the fragments should permit the identification of active and deactivating moieties. The validity of the procedure was established by determining the structural requirements for the carcinogenicity of PAHs and *N*-nitrosamines.[58]

Our data base, summarized in Table 1 consisted of 53 nitroarenes of which 26 were mutagenic (10 or more mutants per nanomole), 22 were inactive (less than 1 mutant per nanomole), and 5 were designated as marginally active (1 to 10 mutants per nanomole).

The results of the analyses allowed recognition of two active fragments (I and II, Figure 6) and two deactivating fragments (III and IV, Figure 6) which appeared to determine the mutagenicity of nitroarenes. Superimposing these fragments upon the nitroarenes reported to be mutagenic (Figure 7) leads to the realization that only one of the chemicals (6-nitrochrysene) was misclassified by the CASE prediction. Similarly, superimposing the relevant fragments on the nonmutagenic nitroarenes (Figure 8) indicated that primarily the hexa- and heptacyclic nitroarenes which are inactive are predicted by CASE to possess mutagenicity. It is entirely plausible that these large molecules might be unable to penetrate through the bacterial cell envelope. This is under investigation at this time. Altogether, CASE predicted correctly the activity of 47 of 53 nitroarenes. The predictions are based upon a mutagenicity data base and the assumption is implicit that the experimental results are accurate and do not reflect artifacts such as the possibility that the mutagenicity of some of the chemicals is due to trace impurities of highly mutagenic chemicals such as the presence of dinitropyrene in 2-nitropyrene.[119] It should be noted that the nitroquinolines and nitrocarbazoles listed in Table 1, although not included in Figures 7 and 8, were also predicted accurately, i.e., only 2-nitrocarbazole was found to contain an activating fragment.

It should be noted that the CASE program can be used in a predictive mode as well (Table 10). It must be stressed that the CASE program is self-learning and its predictability improves with the increase of information in the data base. Obviously, the program is not restricted to nitroarenes and mutagenicity in *Salmonella*, but is applicable to many different situations.

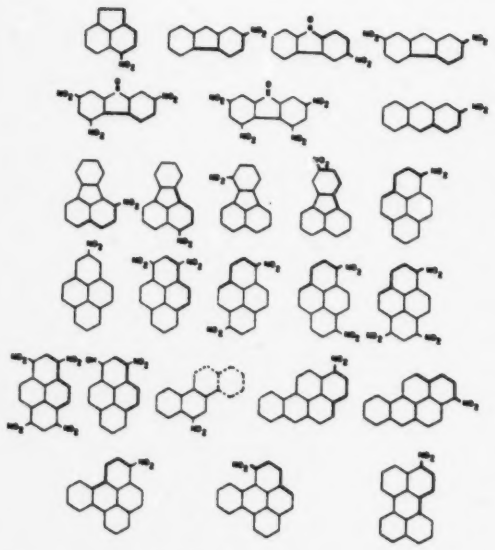

FIGURE 7 Formulae and significant fragments of nitroarenes reported to be mutagenic. The heavy lines indicate active fragments. (Only one such fragment is shown per molecule) The broken lines denote inactivating fragments. Formulae without either such lines have neither active nor deactivating fragments.

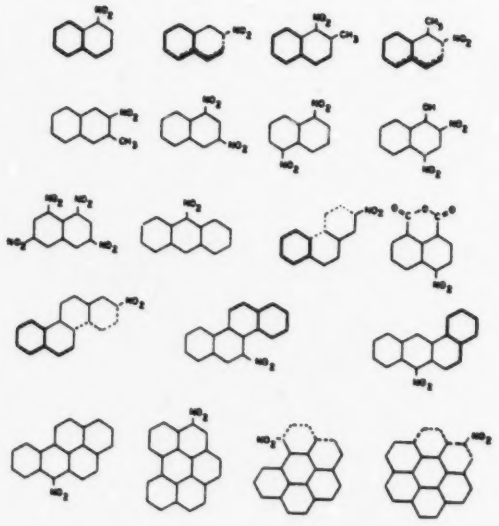

FIGURE 8 Formulae and significant fragments of nitroarenes reported to be nonmutagenic. The heavy lines denote inactivating fragments while the broken lines indicate active fragments.

Table 10
PREDICTED MUTAGENIC ACTIVITIES OF UNTESTED NITROARENES

Compound	Predicted mutagenicity
3-Nitrophenanthrene	Inactive
1-Nitroanthracene	Inactive
1-Nitrotriphenylene	Inactive
2-Nitrotriphenylene	Active
4-Nitropyrene	Inactive
2-Nitrobenzo(*a*)pyrene	Inactive
8-Nitrobenzo(*a*)pyrene	Active
1-Nitroperylene	Active
2-Nitroperylene	Inactive

VI. EVIDENCE FOR A REACTION BETWEEN NITROARENES AND THE ADENINE MOIETY OF DNA

Heretofore, findings have been presented which suggest that the mutagenicity of nitroarenes is related to the ability of their metabolites to react with the guanine moiety of DNA, the arylation occurring primarily at the C-8 position of the guanine. In addition, most of the nitroarenes were found to be powerful mutagens for *Salmonella* tester strains containing guanine residues at the mutagenic site (see Table 8). However, a careful examination of the published literature suggests that adenine could be a target as well. Thus the ability of *N*-hydroxy-2-aminonaphthalene, a metabolite of 2-nitronaphthane, to form 1-(deoxyadenosin-N^6-yl)-2-aminonaphthalene[35-37] has already been alluded to. In addition, *N*-hydroxy-1-aminonaphthalene has been reported to react with polyadenylic acid.[35,36] Similarly, *N*-acetoxy-2-acetylaminofluorene, the model electrophilic metabolite of 2-nitrofluorene and 2-*N*-hydroxy-2-aminofluorene, has been reported to react with the adenine of DNA and of synthetic polynucleotides.[36-40] These findings suggest that "activated" nitroarenes might react with adenine residues and that this may result in a promutagenic event. The recent availability of *Salmonella* tester strains (TA102 and TA96)[41] with adenine at the mutagenic site (Table 8) permitted the determination of whether nitroarenes or their metabolites are capable of forming biologically significant adenine adducts.

Most of the nitroarenes tested, as well as their proximate metabolites, exhibited mutagenicity for the two tester strains which have adenine at the mutational target (Table 11). The relatively high mutagenicity of the nitropyrenes and 3-nitrofluoranthene in these tester strains is very striking. Since it has been shown that with most of the active nitroarene metabolites tested adenine is at best a minor target for adduct formation, it must be argued that if mutagenicity in these strains reflects attack on the adenine, then the promutagenic adenine adduct that is formed by these nitroarene metabolites must contribute a disproportionately large share to the total mutagenicity (i.e., compare the activities of specific chemicals in strains TA98 and TA100 with those of the same agent in strains TA96 and TA102 (Table 11). An analysis of the relationship of the mutagenic potency of these chemicals to the half wave potential revealed a linear plot for the logarithm of the mutagenicity of the nitroarenes in strains TA96 and TA102 vs. the voltage of the half wave potential (Figure 9). The relationship obtained with the same chemicals in strain TA100 is included for comparison; it too shows a linear response (see also Figure 4). These findings indicate that the reaction between the nitroarene metabolites and the putative adenine target is also

Table 11
MUTAGENICITY OF NITROARENES AND PROXIMATE METABOLITES ON *SALMONELLA* STRAINS WITH A-T BASE PAIRS AT THE MUTATIONAL HOT SPOT

Chemical	Revertants/μg			
	TA102	TA98	TA100	TA98
1-Nitronaphthalene	0	0.8	6	0.1
N-Hydroxy-1-aminonaphthalene	0.6	26	57	24.3
2-Nitronaphthalene	0.2	1.0	8	0.7
N-Hydroxy-2-aminonaphthalene	~14	12.4	94	23.4
2-Nitrofluorene	0.3	11	46	8.1
2-Nitrosofluorene	33	87	78	1,592
N-Hydroxy-2-aminofluorene	45	102	138	2,974
1-Nitropyrene	37	2,382	340	1,835
1,3-Dinitropyrene	650	27,580	37,260	496,000
1,6-Dinitropyrene	7,084	136,360	18,585	630,000
1,8-Dinitropyrene	1,240	100,440	19,000	870,000
1,3,6-Trinitropyrene	845	95,100	3,726	60,000
1,3,6,8-Tetranitropyrene	17	10,351	343	10,820
3-Nitrofluoranthene		1,479	418	10,530

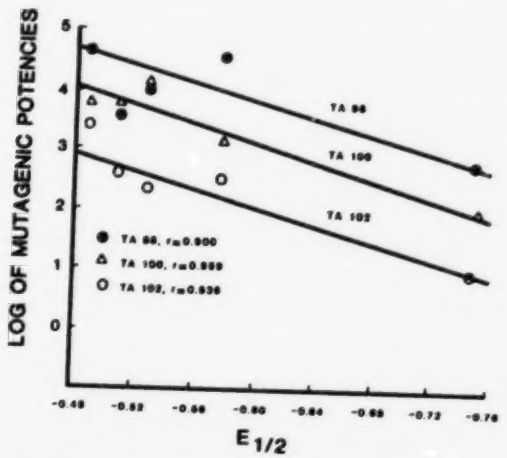

FIGURE 9 Relationship between the mutagenicity of nitropyrenes in strains TA98, TA100 and TA102 and the experimental determined voltage ($E^{1}/_{2}$) of the first half wave potential (i.e., reduction of the first nitro group)

dependent upon, or greatly influenced by, the ease of reduction of the nitro function. In addition, a plot of the logarithm of the mutagenic potencies against the calculated LUMO energies revealed (Figure 10) linear relationships as well. This suggests that the mutagenic

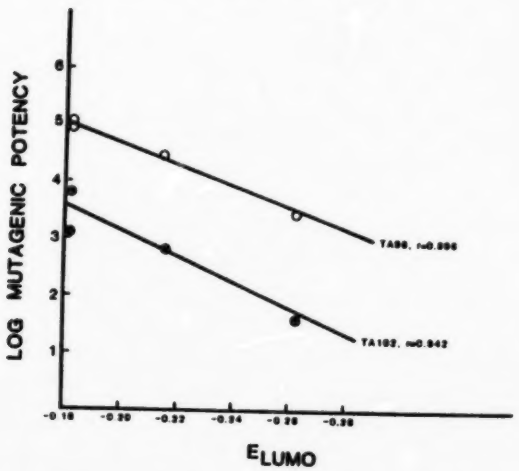

FIGURE 10 Relationship between the mutagenicity of nitroarenes in strains TA96 and TA102 and calculated E_{LUMO} energies in the lowest unoccupied orbital.

potencies of yet untested nitroarenes due to reaction with adenines can be estimated. As the ability to form adducts with adenine might contribute appreciably to the total mutagenic burden induced (as evidenced by the elevated mutagenicity of nitroarenes in strain TA96), it might be of practical importance to be able to determine the total potential mutagenicity capable of being induced by nitroarenes from calculations of the LUMO energies contributed by adduct formation with guanine (i.e., activity in TA98, see Figure 5) and with adenine (i.e., activity in TA96, see Figure 10).

VII. THE EFFECT OF NUCLEOTIDE SEQUENCE AT THE MUTATIONAL HOT SPOT ON THE EXPRESSION OF THE MUTAGENICITY

Biochemical as well as mutagenicity studies have indicated that the guanine residue in DNA is the preferred site for adduct formation with nitroarene metabolites. Indeed, as mentioned previously, the mutagenic potency of these chemicals has been related to the extent of arylation of the C-8 position of the guanine.[23,35] In the course of studies seeking to relate the structure of nitroarenes and their metabolites to their biological activities, it was found unexpectedly that the mutagenicity of *N*-acetoxy-2-acetylaminofluorene (*N*-acetoxy-2-AAF), the model ultimate mutagen, was much less than that of the penultimate *N*-hydroxy-2-aminofluorene (see Table 4). This led to a further investigation of this phenomenon on the assumption that it probably reflected a configurational change resulting from the adduct that was formed between the C-8 of guanine and *N*-acetoxy-2-AAF: *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (G-AAF). This adduct differs from the one formed between *N*-hydroxy-2-aminofluorene and guanine: *N*-(deoxyguanosin-8-yl)-2-aminofluorene (G-AF). The G-AF adduct is in the usual *anti* configuration while the acetyl group in G-

Table 12
MUTAGENICITY OF *N*-HYDROXY-2-AMINOFLUORENE, *N*-ACETOXY-2-ACETYLAMINOFLUORENE, AND DERIVATIVES IN TA98

Chemical	Revertant/nmol
N-Hydroxy-2-aminofluorene	1185
N-Acetoxy-2AAF	21
N-Acetoxy-7-iodo-2AAF	83
N-Acetoxy-2AAF + deacetylase	224

Note: AAF = acetylaminofluorene. The effectiveness of the deacetylation was ascertained by HPLC analysis.

AAF forces the nucleotide into the *syn* configuration. When the *syn* G-AAF adduct is in the midst of alternating guanine-cytosine (dG·dC) sequences, this in turn may result in a shift from the right-handed B-DNA to the left-handed Z-DNA conformation.[52-58] Such alternating dG·dC (and dG·dT) sequences exist not only in eukaryotic DNA,[59-62] but they have also been introduced into bacterial plasmid.[63,64] In fact, they are the mutational target of *Salmonella* strain TA98 (Table 8). Thus the possibility exists that the decreased mutagenicity of *N*-acetoxy-2-AAF in TA98 reflects a change not only from the *anti* to the *syn* configuration, but actually also a shift from a right-handed to a left-handed DNA helix. *N*-Acetoxy-2-acetylamino-7-iodofluorene (iodo-*N*-acetoxy-2AAF) also forms an adduct at the C-8 of the guanine. However, unlike G-AAF, the bulkiness of the iodo atom prevents the shift from the *anti* to the *syn* form,[55,56] and this in turn also precludes formation of the Z-DNA structure even should guanine substitution with the iodo-*N*-acetoxy-2AAF occur in the midst of alternating dG·dC sequences. Iodo-*N*-acetoxy-2AAF showed an increase in the mutagenicity of TA98 when compared with the activity of *N*-acetoxy-2AAF (Table 12). This is in agreement with the hypothesis that the *syn* conformation (with or without shift to the Z-DNA) results in reduced mutagenicity. This notion is confirmed further by the enzymic deacetylation of *N*-acetoxy-2AAF and demonstration of an increase in the mutagenicity of the deacetylated product (Table 12).

The findings discussed above suggest not only that the nature of the base which forms the adduct with DNA (guanine in this instance) but also the sequence in which it finds itself may influence the outcome of the mutational event. This "neighbor effect" may affect the accessibility of the nucleophile (guanine) to the approaching mutagen, or it may involve conformational changes such as shifts from *anti* to *syn* or from right-handed B-DNA to left-handed Z-DNA. To study this further we used the recently developed strain TA97[57] which like TA98 detects frameshift mutations. However, unlike TA98 which has eight alternating dG·dC pairs at the mutational target, TA97 has a run of 6-guanine at that site (Table 8). In this arrangement, chemicals such as *N*-acetoxy-2AAF can still form the G-AAF adduct which, in turn, results in a shift to the *syn* configuration. However, contrary to the situation which exists when G-AAF is in the midst of an alternating dG·dC sequence, the presence of a guanine run precludes the formation of the Z conformation. It was felt that a comparison of the activities of nitroarenes and their metabolites in TA98 and TA97 might yield clues relating to the type of structural change the mutagens were capable of inducing. The results of these studies (Table 13) revealed that *N*-hydroxy-2-aminonaphthalene and *N*-hydroxy-4-aminobiphenyl seem to be mutagenically equipotent in both strains. It is of some interest that these chemicals have been shown[15] to arylate not only the C-8 of guanine, but to form adducts at the guanine-N^2 (i.e., *N*-hydroxy-4-aminobiphenyl and *N*-hydroxy-2-aminona-

Table 13
RELATIVE MUTAGENICITY OF NITROARENES IN TWO *SALMONELLA* TESTER STRAINS DIFFERING IN THE GUANINE-CYTOSINE SEQUENCE AT THE MUTATIONAL SITE

Chemical	TA98/TA97
2-Nitrosofluorene	3.6
N-Hydroxy-2-aminofluorene	3.6
N-Acetoxy-2-acetylaminofluorene	11.0
N-Acetoxy-7-iodo-2AAF	2.7
5-Nitroacenaphthene	2.1
1-Nitropyrene	2.2
1,3-Dinitropyrene	1.6
1,6-Dinitropyrene	1.4
1,8-Dinitropyrene	2.0
1,3,6-Trinitropyrene	2.2
1,3,6,8-Tetranitropyrene	2.8
3-Nitrofluoranthene	23.3
N-Hydroxy-4-aminobiphenyl	1.0
N-Hydroxy-2-aminonaphthalene	1.3
4-Nitroquinolone-1-oxide	1.6

phthalene), the adenine-C8 (i.e., *N*-hydroxy-4-aminobiphenyl), and the adenine-N^6 (i.e., *N*-hydroxy-2-aminonaphthalene). 4-Nitroquinoline-1-oxide, like the above reacts at several sites including guanine and adenine.[58] Hence, it also has more degrees of freedom; it exhibits a ratio of 1.6. On the other hand, 2-nitrosofluorene and *N*-hydroxy-2-aminofluorene, both of which have been reported to form adducts only at the C-8 of guanine, have ratios of 3.6. Significantly, *N*-acetoxy-2AAF, the chemical which forms the *syn* G-AAF adduct, is much less effective as a mutagen for TA97, i.e., ratio is 11.0. This suggests further that it is not only the *syn* configuration which is required for maximal mutagenicity of *N*-acetoxy-2AAF, but also the formation of the left-handed Z-DNA structure. Of course, this cannot occur in TA97.

The nitropyrenes exhibit ratios in the range of 1.4 to 2.8 which does not suggest unusual configurations in spite of their extraordinarily potent mutagenicities. This was also the conclusion that had been reached upon an analysis of the potentiometric reduction (see above). On the other hand, 3-nitrofluoranthene, which has a mass identical to that of 1-nitropyrene, shows a ratio (23.3) which is even larger than that of *N*-acetoxy-2AAF (Table 13), thus suggesting an unusual structural feature that warrants further exploration.

VIII. GENE MUTATIONS AND GENOTOXIC EFFECTS INDUCED BY NITROARENES IN MAMMALIAN CELLS

The ability of nitroarenes to induce mutations in mammalian cells is documented for only eight members of the group, six of them nitropyrenes (Table 14). Both 2-nitrofluorene and 2,4,7-trinitro-9-fluorenone induce mutations in mouse lymphoma cells at the thymidine kinase locus. On the other hand, the results obtained with the nitropyrenes vary. In the absence of metabolic activation, 1-nitropyrene is inactive in Chinese hamster lung fibroblast (CHL) and V79 cells while it is marginally active in Chinese hamster ovary (CHO) cells. It should be mentioned, however, that the markers tested in the CHL and V79 cells are diphtheria toxin and ouabain resistance, respectively. These mutations are thought to be

Table 14
GENE MUTATIONS IN CULTURED MAMMALIAN CELLS

Chemical	Response	Activation	Cell	Genetic marker	Ref.
2-Nitrofluorene	+	Not required	Mouse lymphoma L5178Y	TK	90
2,4,7-Trinitro-9-fluorenone	+		Mouse lymphoma L5178Y	TK	91
1-Nitropyrene	+	Required	Mouse lymphoma L5178Y	TK	92, 93
1,8-Dinitropyrene	+	—	Mouse lymphoma L5178Y	TG, MTX, OUA, AraC	94
1-Nitropyrene	–	Absent	Chinese hamster ovary (CHO)	TK	92
1-Nitropyrene	+ (marginal)	Not required	Chinese hamster ovary (CHO)	HGPRT	95
1-Nitropyrene	+	Enhanced by S9	Chinese hamster ovary (CHO)	HGPRT	95
1,3-Dinitropyrene	+ (marginal)	Not required	Chinese hamster ovary (CHO)	HGPRT	95
1,6-Dinitropyrene	+	Not required	Chinese hamster ovary (CHO)	HGPRT	95
1,8-Dinitropyrene	+	Not required	Chinese hamster ovary (CHO)	HGPRT	95
1,3,6-Trinitropyrene	+	Not required	Chinese hamster ovary (CHO)	HGPRT	95
1-Nitropyrene	–	Absent	Chinese hamster lung fibroblast	Diphtheria toxin	96
1,3-Dinitropyrene	+	Absent	Chinese hamster lung fibroblast	Diphtheria toxin	96
1,6-Dinitropyrene	+	Absent	Chinese hamster lung fibroblast	Diphtheria toxin	96
1,8-Dinitropyrene	+	Absent	Chinese hamster lung fibroblast	Diphtheria toxin	96
1,3,6-Trinitropyrene	+	Absent	Chinese hamster lung fibroblast	Diphtheria toxin	96
1,3,6,8-Tetranitropyrene	–	Absent	Chinese hamster lung fibroblast	Diphtheria toxin	96
1-Nitropyrene	–	Absent	Chinese hamster V79	OUA	97
1-Nitropyrene	+	Irradiated Syrian hamster cells	Chinese hamster V79	OUA	97
1,3-Dinitropyrene	+	Not required	Chinese hamster V79	OUA	97
1,8-Dinitropyrene	+	Not required	Chinese hamster V79	OUA	97
1,8-Dinitropyrene	–	Absent	(Human) XP	HGPRT	98
1,8-Dinitropyrene	+	Absent	Human diploid lymphoblasts	OUA, TG	99

induced as a result of base substitution mutations. Paradoxically, 1-nitropyrene is a frameshift mutagen in *Salmonella*. The marker tested in the CHO cells was the HPGRT locus, which detects both frameshift and base substitution mutations. It is conceivable, therefore, that the genetic loci which detect base substitution mutations have a higher response threshold for nitroarenes and hence register only borderline mutagenicity for 1-nitropyrene. Thus, a locus capable of detecting base substitution as well as frameshift mutations might be more responsive to the mutagenic action of 1-nitropyrene. The addition of an activation system (S9 or irradiated Syrian hamster embryo cells) resulted in the unequivocal demonstration of the mutagenicity of 1-nitropyrene (Table 14).

1,3-, 1,6-, and 1,8-Dinitropyrenes were directly mutagenic in all of the assay systems including Chinese hamster, murine, and human cells (with the exception that 1,8-dinitropyrene lacked demonstrable activity for xeroderma pigmentosum cells). Various genetic loci were tested including diphtheria and ouabain resistances. 1,3,6,8-Tetranitropyrene was tested only in CHL cells with diphtheria toxin resistance as the marker. In that system it did not exhibit any mutagenic activity. Perhaps its situation is similar to that of 1-nitropyrene (see above).

In addition to inducing gene mutations, nitroarenes also induce a variety of genotoxic effects (Table 15) including unscheduled DNA synthesis (UDS), chromosomal aberrations, and sister chromatid exchanges (SCE) in vivo as well as in vitro (Table 15). The potency of these effects varies depending upon the nature of the target cells and exogenous metabolic activation required.

Unfortunately, unlike the situation that exists with the *Salmonella* mutagenicity data (Table 1), the number of nitroarenes tested for gene mutations and genotoxicity in mammalian cell systems is small which, in turn, limits the usefulness of an artificial intelligence program such as CASE to determine common structural determinants of biological activity. Moreover, an examination of the results (Tables 14 and 15) suggests that the metabolic capabilities of the different cells may affect the outcome of the genetic tests. Accordingly, it is quite possible that some of the cells used in these assays may have lost, upon continued passage, their capacity to activate and/or detoxify chemicals and therefore may not adequately reflect the situation of the tissue of origin. It would thus seem that the study of the metabolic conversion of nitroarenes in whole cells or cell-free microsomal preparations will be necessary to fully understand the mutagenic and carcinogenic potentials of this group of chemicals.

IX. METABOLISM OF NITROARENES BY MAMMALIAN ENZYMES

The standard methods for demonstrating the mutagenicity of PAHs is to incorporate a microsomal preparation (more usually a post-mitochondrial preparation called S9) together with co-factors into the bacterial mutagenicity assay. Indeed, nitroarenes are also metabolized by such preparations, however, the products formed are not always mutagenic for bacteria. This appears to be due to the fact that the metabolites capable of exerting mutagenicity in mammalian cells are not necessarily identical to those mutagenic for bacteria. However, a combination of appropriate bacterial tester strains fortified with S9 preparations can help dissect the situation and lead to the identification of metabolites that are active in mammalian cells. Thus, nitroreductase-deficient bacterial strains may be quite useful when combined with S9 in elucidating bioactivation pathways of nitroarenes that are unique to mammalian cells. Several examples follow.

5-Nitroacenaphthene. A combination of such procedures was most useful in the study of the mutagenic bioconversion products of 5-nitroacenaphthene. As a matter of fact, it led to the development of a simple procedure for recognizing and differentiating between bacterial and mammalian mutagenic metabolites of 5-nitroacenaphthene.[59] It was first realized (see Table 16) that the mutagenic specificity of 5-nitroacenaphthene was different depending

Table 15
GENOTOXIC EFFECTS OF NITROARENES

Chemical	Genetic end point	System	Activation	Result	Ref.
1-Nitropyrene	UDS	Primary rat hepatocyte	None	+	92
1,6-Dinitropyrene	UDS	Rat hepatocytes	None	+	100
1-Nitropyrene	UDS	Human bronchus	None	+	101
1,3-Dinitropyrene	UDS	Human bronchus	None	+	101
1,6-Dinitropyrene	UDS	Human bronchus	None	+	101
1,6-Dinitropyrene	UDS	Human hepatocytes	None	+	100
Nitrated pyrenes*	UDS	HeLa	None	+	102
Nitrated fluoranthenes*	UDS	HeLa	None	+	102
Nitrated perylenes*	UDS	HeLa	None	+	102
Nitrated chrysenes*	UDS	HeLa	None	+	102
Nitrated anthracenes*	UDS	HeLa	None	+	102
Nitrated benzo(*a*)pyrenes*	UDS	HeLa	None	+	102
Nitrated benzo(*e*)pyrenes*	UDS	HeLa	None	+	102
Nitrated benz(*a*)anthracenes*	UDS	HeLa	None	+	102
Nitrated benzo(*g,h,i*)perylenes*	UDS	HeLa	None	+	102
1,6-Dinitropyrene	Chromosome aberrations	Rat epithelial cell line	None	+	103
1,8-Dinitropyrene	Chromosome aberrations	Rat epithelial cell line	None	+	103
1,6-Dinitropyrene	Chromosome aberrations	Human fibroblast cell line	None	Marginal	104
1,8-Dinitropyrene	Chromosome aberrations	Human fibroblast cell line	None	Marginal	104
1,8-Dinitropyrene	Preferential toxicity	Human XP cells	None	–	98
2-Nitrofluorene	Inhibition of DNA synthesis	HeLa	None	–	105
2-Nitrofluorene	SCE	CHO	Rat S9	+	106
1-Nitropyrene	SCE	CHO	Rat S9	+	106
1,8-Dinitropyrene	SCE	CHO	Rat S9	+	106
2,4,7-Trinitro-9-fluorenone	SCE	CHO	Rat S9	+	91
1-Nitropyrene	In vivo SCE	Rat (bone marrow)		+	107
2-Nitrofluorene	Leukemia viral enhancement		None	+	108

Abbreviations: SCE sister chromatid exchanges, CHO, Chinese hamster ovary cells, UDS, unscheduled DNA synthesis.

* Mixtures

Table 16
MUTAGENICITY OF 5-NITROACENAPHTHENE IN THE PRESENCE AND ABSENCE OF S9

S9	TA100	TA98
None	9.8	12.4
RI	70.6	21.5
MI	57.3	30.7
HI	283	151
None	0.14	0.58
RI/RI	1.00	1.00
MI/RI	0.81	1.43
HI/RI	4.01	7.02
	TA100/TA98	**TA98/TA98**
None	0.79	1.00
RI	3.28	1.00
MI	1.87	1.00
HI	1.88	1.00

R, rat; M, mouse; H, hamster; I, Aroclor 1254-induced.

upon the presence of S9. Thus in the absence of exogenous activation, the mutagenicity of 5-nitroacenaphthene was primarily of the frameshift variety (Table 16), i.e., the ratio of TA100/TA98 was <1.0. In the presence of S9, however, this activity shifted to a base substitution type, i.e., TA100/TA98 >1.0. This suggests that the adduct formed in the presence as well as the absence of S9 induce different mutagenic events. The S9-mediated formation of the active metabolites was dependent upon the species of origin of the S9, being highest in preparations derived from Aroclor-induced hamsters (i.e., HI/RI >4).

Such findings led us to investigate the possibility of coupling S9 to nitroreductase- or esterification-deficient bacteria. Indeed it was found that S9 allowed the demonstration of the mutagenicity of 5-nitroacenaphthene in these strains (Table 17). Since it is doubtful that S9 is equally capable of supplying nitroreductase and esterification enzyme activities simultaneously, it was concluded that S9 probably caused ring oxidation in a manner similar to that described by El-Bayoumy and Hecht:[60] i.e., the formation of 1- and 2-hydroxy-, and 1- and 2-oxo-nitroacenaphthene derivatives. Because these mutagenic metabolites are active in nitroreductase-deficient strains it is conceivable that the oxygenated intermediates are direct-acting or alternatively (but less likely) that the S9 is supplying a nitroreductase function.

1-Nitropyrene. 1-Nitropyrene appears to be the most abundant nitroarene species present in diesel emissions.[61] As a matter of fact it has been suggested that it be used as a surrogate for the other nitroarenes present therein. The mutagenicity of this chemical is almost entirely abolished in strain TA98NR.[18] However, addition of S9 to 1-nitropyrene incubated in the presence of the nitroreductase-deficient strain TA98NR resulted in the restoration of the mutagenic activity.[62-64] Subsequent studies showed, however, that the activity was not the result of a nitroreductase activity supplied by the S9, but rather to ring oxidation. This led to a number of studies seeking to determine the biotransformation of 1-nitropyrene in microsomal preparations as well as in animals. El-Bayoumy and Hecht[65] have shown that incubation of 1-nitropyrene with rat S9 resulted in the formation of 1-nitropyren-3-ol, 1-nitropyren-6-ol, 4,5-dihydro-4,5-dihydroxy-1-nitropyrene, and 1-aminopyrene. The monohydroxynitropyrenes are more mutagenic than 1-nitropyrene suggesting that they may be

Table 17
S9-MEDIATED MUTAGENICITY OF 5-NITROACENAPHTHENE

	−S9	+S9
TA100	22.1	88.9
TA100NR	4.1	45.4
TA98	23.0	96.8
TA98NR	13.6	87.4
TA98/1,8-DNP$_2$	2.7	59.6
TA98NR/1,8-DNP$_2$	2.5	

Note: Mutagenicities were calculated from the linear portions of the dose response curves. S9 was derived from the livers of Aroclor induced rats.

proximate mutagenic species. When the oxygen tension was reduced, 1-aminopyrene was the major metabolite. Similar studies were also performed by Howard and Beland[74] who recovered 1-nitropyren-8-ol in addition to the 3- and 6-monohydroxylated derivatives of 1-nitropyrene. Small quantities of 1-aminopyrene and *N*-acetylaminopyrene were also detected. Upon intravenous administration of 1-nitropyrene to Sprague-Dawley rats the major biliary metabolites were identified as the *O*-glucuronides of 3-, 6-, and 8-hydroxy-1-nitropyrenes. Reduced aminopyrenes were not detected. Ball and associates[66] administered 1-nitropyrene interperitoneally to rats and recovered metabolites in the urine and feces. Ring hydroxylated derivatives of 1-nitropyrene and *N*-acetylaminopyrene were recovered, primarily as the glucuronides.

In comparing the metabolism of 1-nitropyrene to that of unsubstituted PAHs it should be noted that no dihydrodiol was formed at the 9,10- positions of 1-nitropyrene. Perhaps the presence of the nitro group adjacent to the *peri* position inhibits the formation of such a dihydrodiol. Such has also been found to be the case with 6-nitrobenzo(*a*)pyrene[67] and 6-nitrochrysene.[68]

1,8-Dinitropyrene. 1,8-Dinitropyrene is mutagenic in a number of mammalian systems while being without activity in others (see Tables 14 and 15, e.g., xeroderma pigmentosum). Since it is known that upon continued passage cells tend to lose microsome-associated enzymes such as the monooxygenases, it is doubtful that the lack of activity of dinitropyrene in such systems is due to loss of a nitroreductase activity. As a matter of fact, in one system (unscheduled DNA synthesis in CHO cells) addition of S9 results in demonstration of UDS. On the other hand, addition of S9 derived from the livers of rats, mice, or hamsters to 1,8-dinitropyrene results in an almost quantitative loss of mutagenic activity.[69] The S9 preparations derived from Aroclor 1254-treated animals were even more efficient than those derived from uninduced animals in abolishing this mutagenicity. Recent studies have shown (Table 18) that the inhibitory effect of S9 derived from the livers of Aroclor-induced hamsters could be greatly inhibited by 7,8-benzoflavone, an inhibitor of cytochrome P_{450},[70] a species induced by Aroclor 1254[71,72] (other microsomal inhibitors had no significant effects; see Table 18). By coupling inhibitors of microsomal enzymes with tester strains deficient either in specific nitroreductases or in esterification enzymes it is hoped that the nature of the ultimate mutagenic species of 1,8-dinitropyrene in mammalian cells can be unraveled.

6-Nitrobenzo(*a*)pyrene. 6-Nitrobenzo(a)pyrene is not a direct-acting mutagen for *Salmonella typhimurium* TA98 (Table 1). The demonstration of the mutagenicity of this chemical requires the participation of microsomal enzymes.[73] Studies on the microsome-mediated

Table 18
EFFECT OF INHIBITORS OF MICROSOMAL ENZYMES ON THE S9-MEDIATED INHIBITION OF THE MUTAGENICITY OF 1,8-DINITROPYRENE

Additions	Revertants/μg	
	−S9	+S9
1,8-Dinitropyrene	601,194	12,733
1,8-Dinitropyrene + Metyrapone (10^{-3})	400,552	13,382
1,8-Dinitropyrene + 7,8-Benzoflavone (200 μM)	426,824	88,044
1,8-Dinitropyrene + Cytochrome *c* (300 μM)	448,067	17,054

Note: Mutagenicities were calculated from the linear portion of the dose-response curves.

metabolism of 6-nitrobenzo(a)pyrene have shown[67] that 3-hydroxy-6-nitrobenzo(*a*)-pyrene was a major metabolite with 6-nitrobenzo(*a*)pyrene-3,9-hydroquinone, 1-hydroxy-6-nitrobenzo(*a*)pyrene, and 6-nitrobenzo(*a*)-pyrene-1,9-hydroquinone as additional products. Chou et al.[74] have established the existence of a 2,3-epoxide intermediate which spontaneously rearranges via an NIH shift to yield the 3-hydroxy-6-nitrobenzo(*a*)pyrene. It is of interest to note that unlike benzo(*a*)pyrene, which in addition to oxidation to 3-hydroxybenzo(*a*)pyrene also oxidizes at the 4,5-, 7,8-, and 9,10-regions the presence of the nitro group at the 6 position inhibits metabolism at the region *peri* to the nitro function.

In hamster embryo cells, dihydrodiol derivatives appear to be the major metabolic species of 6-nitrobenzo(*a*)-pyrene. Phenols are also present but as glucuronides.[75]

X. PERSPECTIVE ON THE METABOLISM OF NITROARENES IN MAMMALIAN SYSTEMS

The several studies outlined above indicate that whereas the metabolism of nitroarenes in bacteria proceeds primarily via reduction of the nitro moiety (Figure 1), in mammalian cells nitroreduction may be an unlikely pathway in the presence of oxygen. Rather, it would appear (Figure 11) that in mammalian cells the primary pathway is ring oxidation. The nitro group, by blocking *peri* oxidation, has a directing effect. Two major pathways appear to be operative: (1) oxidation of the ring which may then be followed by nitroreduction to the arylhydroxylamine and ultimately by the formation of an active arylnitrenium ion and (2) ring oxidation, possibly via epoxides not followed by nitroreduction. The oxygenated ring derivative may be the ultimate reactive species presumably being capable of forming DNA adducts. Indeed, the studies with 5-nitroacenapthene in bacteria in the presence of S9 indicate that the adduct formed when ring oxidation occurs is quite different from the one formed in the absence of mammalian enzymes (and probably does not involve nitroreduction). Finally, it should be noted that in a number of instances nitroreduction was not observed as evidenced by the presence of nitro-containing oxidized PAHs (i.e., 6-nitrobenzo(*a*)pyrene metabolites). If it be argued that formation of the ultimate DNA adduct requires reaction with the arylnitrenium ion (because the ring oxidized species are incapable of forming adducts) and that nitroreduction does not occur in the mammalian cells under study, then the role of the anaerobic bacterial flora must be considered. It has been demonstrated on a number of occasions that the anaerobic flora of the colon is metabolically very active and is capable of reducing the nitro function.[22,76-78]

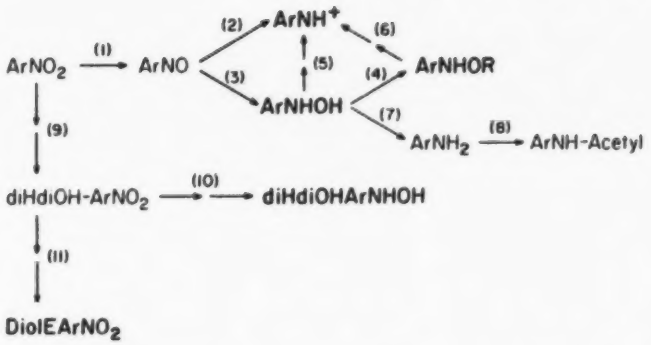

FIGURE 11. A scheme describing the metabolism of nitroarenes in mammalian systems. Nitroreduction can follow the same pathway as in bacteria (see Figure 1) or it can involve ring oxidation which may be followed by nitroreduction. In the whole animal, the final nitroreduction of the ring oxygenated nitroarene may involve nitroreduction by the anaerobic bacterial flora. (The bold-lettered intermediates are thought to be ultimate mutagens and/or DNA reactants.)

Table 19
CELL TRANSFORMING ACTIVITY OF NITROARENES

Chemical	System	Activation required	Result	Ref.
2-Nitrofluorene	Hamster BHK 21	+ S9	+	109
2-Nitrofluorene	Syrian hamster embryo	Hamster hepatocytes	+	110
2-Nitronaphthalene	Syrian hamster embryo	None	+	110
1-Nitropyrene	Syrian hamster embryo	None	+	111
1,8-Dinitropyrene	Syrian hamster embryo	None	+	111
3-Nitrofluoranthene	Syrian hamster embryo	None	+	111
6-Nitrochrysene	Syrian hamster embryo	None	+	111
6-Nitrobenzo[*a*]pyrene	Syrian hamster embryo	None	+	111
1-Nitropyrene	Mouse Balb 3T3	None		67
1,3-Dinitropyrene	Mouse Balb 3T3	None		67
1,6-Dinitropyrene	Mouse Balb 3T3	None		67
1,8-Dinitropyrene	Mouse Balb 3T3	None		67
1,3,6-Trinitropyrene	Mouse Balb 3T3	None		67
1,3,6,8-Tetranitropyrene	Mouse Balb 3T3	None		67
1-Nitropyrene	Normal human fibroblasts	Anaerobiosis	+	24
6-Nitrobenzo[*a*]pyrene	Normal human fibroblasts	Anaerobiosis	+	24

XI. NEOPLASTIC CELL TRANSFORMING ACTIVITY OF NITROARENES

Except for the lack of activity of the nitropyrenes in the mouse Balb 3T3 system (Table 19), all of the nitroarenes tested induced cell transformations as measured by focus formation or anchorage independence. As with the gene mutations and genotoxicity assays, the number of nitroarenes tested thus far is too small to permit determination of the structural determinants of cell transforming activity. Hopefully, as more nitroarenes are assayed in these various short-term assay systems it will be possible to conduct such studies.

Table 20
CARCINOGENICITY OF NITROARENES

Chemical	Species	Administration	Result	Ref.
5-Nitroacenaphthene	Rat, mouse	Feed	Distal tumors	112
	Rat, mouse, hamster	Feed	Distal tumors	113
1-Nitronaphthalene	Rat, mouse	Feed	Negative	112
2-Nitronaphthalene	Dog, monkey	Feed	Bladder tumors	114
2-Nitrofluorene	Rat	Feed	Distal tumors	115
1-Nitropyrene[a]	Rat	Subcutaneous	Sarcomas (histiocytomas) at site of injection	116
1-Nitropyrene	Rat	Subcutaneous	Tumors at site of injection and mammary tumors	117
1-Nitropyrene	Mouse	Skin + promotion	Negative	118
1,3-Dinitropyrene	Rat	Subcutaneous	Spindle cell sarcomas at site of injection	119
1,8-Dinitropyrene	Rat	Subcutaneous	Spindle cell sarcomas at site of injection	119
3-Nitrofluoranthene	Rat	Subcutaneous	Sarcomas (histiocytomas) at site of injection	116
6-Nitrofluoranthene	Mouse	Skin + promotion	Skin tumors	118
6-Nitrochrysene	Mouse	Skin + promotion	Skin tumors	118
6-Nitrobenzo(*a*)pyrene	Mouse	Skin + promotion	Negative	118
3-Nitroperylene	Mouse	Skin + promotion	Skin tumors	118

[a] This preparation of 1-nitropyrene contained 0.4% of each of the three isomeric dinitropyrenes (T. Sugimura, personal communication, 1983).

XII. THE CARCINOGENICITY OF NITROARENES

A number of nitroarenes are currently being assayed for their ability to induce cancers in rodents. Thus far only 1-nitronaphthalene has consistently given negative results in the standard carcinogenicity bioassay. 1-Nitropyrene has proven to be negative in the two-stage mouse skin carcinogenicity assay, however, the same chemical when administered subcutaneously to rats has been shown to induce tumors not only at the site of injection but at distal sites (mammary tumors) (Table 20). The data (Table 20), though still sparse, do indicate that the nitroarenes as a group are capable of inducing tumors in animals. Preliminary calculations of the carcinogenic potency of the dinitropyrenes indicate that on a weight basis, they have the same activity as benzo(*a*)pyrene in inducing subcutaneous tumors at the site of injection in rats. This indicates that their mutagenic potencies in microbial systems are not reflected in their carcinogenic potentials. This is reassuring in view of the wide distribution of these chemicals in the environment.

XIII. A PERSPECTIVE ON THE NITROARENES

As has been stressed throughout this presentation, there are hundreds of nitroarenes in the environment and we possess biological information on only very few of these. Obviously, since it is neither practical nor feasible to prepare sufficient quantities of each of the environmental nitroarenes to test them in various systems, we will be limited in studying the genotoxicity, mutagenicity, biochemistry, and carcinogenicity of only a few surrogates. This makes the development of predictive procedures based on sound structure-activity consid-

erations important. We have presented herein two approaches (CASE and LUMO energy determinations) that we are developing. Undoubtedly other possibilities are also available and are being pursued by others.

In assessing the risk posed by environmental nitroarenes, we have mentioned neither the route of entry, nor their bioavailability, nor their ensuing tissue distribution. The major entry mode of nitroarenes is by inhalation. However, environmental nitroarenes can be present in the atmosphere adsorbed onto various particles such as fly ash, diesel emissions, and carbon black, or they may be present in the gaseous phase of sidestream cigarette smoke and automobile exhausts. Obviously, the ultimate dose of nitroarenes delivered to tissues will depend upon their desorption from the particles. It is certain that this will not be identical for fly ash, diesel materials, and carbon black. Obviously the physical state of the nitroarene will play a determining role in its tissue distribution. Preliminary studies with 1-nitropyrene have shown that an aerosol of pure 1-nitropyrene (presumably as it occurs in the gaseous phase) is excreted primarily in the urine. On the other hand, when it is adsorbed onto a synthetic particle of respiratory size it is excreted in the feces. Upon desorption from the particle, the nitropyrene can be found bound to macromolecules in the kidneys and liver thus suggesting that studies in whole animals should not be restricted to the lungs.

In this connection, it should be recalled that even though some of the ring oxidized nitroarenes may retain unreduced nitro groups, the intestinal flora is capable[72,76,78] of reducing these to active arylhydroxylamines and further to arylamines. The carcinogenicity of the latter, especially in the bladder is a well recognized phenomenon.

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